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Patentanmeldung Nr. | Patent application No. | Demande de brevet n°

99201301.1

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Blatt 2 der Bescheinigung
Sheet 2 of the certificate
Page 2 de l'attestation

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Bezeichnung der Erfindung: Title of the invention: Titre de l'invention:

Identification of ligands for orphan receptors using on-line coupling of mass spectrometry to continuous-flow separation techniques

In Anspruch genommene Priorität(en) / Priority(ies) claimed / Priorité(s) revendiquée(s)

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Bemerkungen: Remarks: Remarques:

See for riginal title page 1 of the description.

Title: Orphan affinity protein assay using on-line coupling of mass spectrometry to continuous-flow separation techniques.

The present invention relates to the on-line coupling of mass spectrometry (MS) to continuous-flow separation techniques for detecting 'orphan' analytes, viz. affinity molecules which do not have known ligands, such as orphan affinity proteins, e.g. orphan receptors. In a further embodiment, this on-line detection method is used as either a screening method for assays with an unknown ligand. Furthermore, this invention relates to compounds detected by this method and the use of these compounds as a ligand for affinity molecules.

A range of biochemical assays based on continuous flow biochemical detection has been developed during the recent years. Typically, the affinity interaction between ligand and affinity protein has been monitored by measuring the concentration of highly sensitive, usually fluorescence labeled reporter molecules using either heterogeneous or homogeneous biochemical detection strategies. When dealing with 'orphan' receptors however, i.e. receptors for which 'affinity' ligands have not been found so far, new strategies have to be developed, which enable so-called 'label-free detection' of affinity ligands. In contrast to the label-based assays, which usually allow the non-binding analytes from the sample to co-elute with the reporter molecules, label-free assays for orphan receptors demand strict separation of both binding and non-binding ligands.

By configuring restricted access columns and hollow fiber modules in the biochemical part of the system, affinity ligands can be isolated from complex biological matrices. Subsequently, coupling the biochemical setup to mass spectrometry allows the on-line detection of ligands exhibiting affinity for the orphan affinity molecule.

It is known from the prior art that biochemical assays are highly sensitive detection techniques, which

chromatographic or other fractionation systems, since these techniques do not allow the continuous monitoring of the fractionation effluent.

In the article of Irth et al. in the Journal of Chromatography 633 (1993) 65-72 and in the articles of Oosterkamp et al. in Anal. Chem. 66 (1994) 4295-4301, and in the Journal of Chromatography 653 (1994) 55-61, a method for the on-line detection of digoxigenin and its metabolites is described. The on-line detection process comprises the direct injection of a sample containing digoxigenin and its metabolites, a liquid chromatographic (LC) fractional separation step, the mixing of the effluent of the LC column with fluorescein-labeled antibodies against digoxigenin, the removal of free labeled antibodies from the mixture via passage through a small column packed with an antigen-bound support, and detection of the strongly fluorescent biochemical complexes.

The digoxigenin system described is based on association reactions of antibodies and antigens eluting from the analytical column. By the use of fluorescein-labelled antibodies detection limits in the nanomolar range are obtained.

This prior art assay is a heterogeneous detection system. It requires a separation step between free and bound label. Examples for free/bound label separation techniques are restricted-access phases and hollow-fibre modules.

The biochemical reagent in the previously described prior art assay consists of fluorescein-labelled fragments of anti-digoxigenin antibodies which were immunopurified and are commercially available. The commercial availability of purified, labelled antibodies is however exceptional. In almost all cases, antibodies are only available in unlabelled state in crudely purified antiserum. Although labelling and purification schemes for antibodies (or other affinity proteins) are known to the person skilled in the art, it will

molecules bind analytes in the effluent, followed by a separation step using a restricted-access support, whereby the analyte-affinity molecule complex is permeated, followed by a suitable dissociation step to dissociate the analyte-affinity molecule complex, followed by a second separation step in which the dissociated analyte and affinity molecules are separated, followed by detection of the analyte using the mass spectrometer.

The use of the first restricted access column provides the permeation of the analyte-affinity molecule complex while the smaller molecules are retained.

The dissociation step results in the dissociation of the complex. By subsequently separating the affinity molecules from the effluent the analyte can be directed to the MS.

In particular, a suitable affinity molecule for analytes to be detected - which affinity molecule may e.g. be an affinity protein - is added to the effluent of a liquid chromatography or a capillary electrophoresis column to react with the analytes eluting from the column.

The second separation step is carried out either by using a restricted-access support or by using a hollow fiber support.

When using a restricted-access support in the second separation step the affinity molecule is retained, followed by elution of the affinity molecule from the restricted-access support using a suitable carrier stream, and directing the eluted stream to the mass spectrometer. Prior to elution of the affinity molecule, a washing step can be carried out to remove the dissociation solvent and the receptor proteins from the restricted-access column. In order to prevent false positives, it is important that the conditions, with respect to concentrations of compounds such as solvents, of the second restricted-access column are substantially constant and substantially the same as in the first restricted-access column.

Further, the problem associated with cross-reactivity does not occur when using the method of the present invention.

The method of the present invention uses bioaffinity molecules such as receptors, to detect any compounds showing high affinity for the ligand binding site of said affinity molecule. The compounds to be detected may be biochemical compounds but are not in any way restricted thereto.

A system of high-throughput screening to be used in drug discovery may, for instance, comprise the following steps. Complex samples generated for instance by an upstream combinational chemistry system are prefractionated in fractions containing compounds of similar polarity using, e.g., a solid-phase extraction technique or electrophoretic sample handling principles. Each fraction may additionally be fractionated using, e.g., either analytical or preparative-scale liquid chromatographic fractionating columns. The compounds eluting from said LC column are on-line detected using a suitable affinity molecule detection technique. Where preparative-scale fractionating columns are applied, a post-column flow-split will be made. One of the two flow streams is subjected to detection using the affinity molecule detection technique; the other stream is directed to a fraction collector. Dependent on the signal obtained from the affinity molecule detector, fractions containing compounds causing a positive response will be collected while fractions causing a negative response will be discarded. This complete screening method can be automated using known valve-switching processes.

A suitable fractionation method to be used in the methods of the present invention comprises a liquid chromatography fractionation or a capillary electrophoresis step. Other fractionation techniques which are known to the person skilled in the art and which allow a relatively continuous output stream can, however, be used as well.

In a preferred embodiment, the liquid chromatography separation step is a reversed phase HPLC step.

experiments to establish the optimal conditions and materials for each case.

As already mentioned, the fractionation step can be a liquid chromatography separation, a capillary electrophoresis step or a combinatorial chemistry system. It has been found that it is very advantageous when this fractionation step is followed by a separation step, for example by using a hollow-fiber module, since this removes the high molecular weight background. Preferred liquid chromatography fractionation steps comprise HPLC, reversed phase HPLC, CE, CEC, IEF or MEKC, all of which techniques are known to the person skilled in the art.

All possible variations in MS techniques known in the art can be profited from according to the present invention. preferably, the MS is of the type chosen from the group consisting of electrospray ionization type, atmospheric pressure ionization type, quadrupole type, triple quadrupoles (QQQ) type, magnetic sector type, time-off-flight type, MS/MS, MS^n, FTMS type, ion trap type and combinations thereof.

For example, in scanning mode to trace compounds, low resolution MS with all possible instrumental designs of MS can be used, in particular quadrupole, magnetic sector, time-off-flight, FTMS and ion-trap. This generates typically molecular weight data with nominal mass accuracy.

When high resolution MS is applied, using all possible high resolution instrumental designs, in particular magnetic sector, time-off-flight, FTMS and ion trap, molecular weight data with high mass accuracy combined with the elemental composition of the compound can be obtained.

Other known MS techniques comprise tandem MS, such as MS/MS or MS^n (for example MS^3). Application of these techniques enables the collection of structural information of the ligands, which is a preferred embodiment of the present invention. The data in scanning mode can be acquired in data-dependent mode which means that for each peak

complex nature, for instance biological fluids or extracts, natural product extracts, solutions or extracts from biotechnological processes, resulting from chemical experiments, such as combinatorial technologies and processes and the like can be performed with a higher efficiency, selectivity and flexibility. Moreover, the present invention provides the possibility to limit the disturbance of background compounds.

The present invention further enables the identification of compounds based on conventional mass spectra, high resolution data or MS^n based spectra.

With the method of the present invention it is also possible to perform library searching, based on a variety of mass spectrometric experiments enabling the screening of large series of samples and classifying these in classes based on similar active compounds, without the need for full identification of the compounds.

The assay method of the present invention can be applied in miniaturized formats of assay-based systems, for instance with chip technology based screening systems.

In the methods of the present invention all molecules capable of interaction with or binding to other molecules can be used as affinity molecule.

On-line coupling as used in the methods of the present invention requires fast reaction times in order to minimize extra-column band broadening. This means that affinity molecule-ligand interactions having reaction times in the order of minutes rather than hours should be considered. The suitable binding conditions under which the affinity molecules bind to the analyte comprise a contact time, which is in the same order of magnitude. Suitable binding conditions are conditions that provide optimal binding between the affinity molecules and the analyte. It will be understood that the precise conditions, such as temperature, residence time, chemical composition, will

fragments. After reaction Fab bound digoxin was able to pass the first restricted access column, whereas excess unbound molecules were trapped onto the first RA-column. After passing the first RA-column the affinity complex was dissociated by a pH shock using a solution of hydrochloric acid. Again the mixture was allowed to react for 30 s after which the ligand could be trapped onto the second RA-column. Three minutes after injection, the second RA-column was flushed for one minute, after which the second RA-column was switched into the desorption solution. Digoxin was desorbed and subsequently detected by the MS.

Figure 1 presents a typical MS spectrum of digoxin. Apart from the protonated molecule (780.8), an ammonium cluster can be observed at 797.9, whereas the m/z ratios of 762.8 and 651.1 represent the loss of respectively water and a sugar group. Wideband activation of the protonated molecule (780.8 +/- 18) at 45% collision energy fragmentates both the parent mass as well as the ammonium complex to 651.1, which can be observed as the base peak in MS/MS spectra under the conditions used (figure 2). During assay operation digoxin was detected using both MS and MS/MS (m/z 797.9 and 651.1 respectively).

Injecting 5, 50 and 500 ng digoxin in the assay system with and without anti digoxigenin Fab fragments showed that digoxin was completely trapped when the affinity protein was absent. However addition of anti digoxigenin Fab fragments showed increasing response of digoxin (measured at m/z 651.1), demonstrating the ability of the system to detect digoxin using the MS-based orphan assay.

In order to check the robustness of the ms-bcd system, the anti-digoxigenin Fab fragment solution was replaced by goat antibiotin. Injection of 0, 5, 50 and 500 ng digoxin showed that the signal obtained for 500 ng is still comparable to the blank, meaning that the excess of digoxin is adequately trapped on the first restricted access column.

Claims

1. On-line detection method comprising the on-line coupling of the effluent of a fractionation step to a mass spectrometer, which method comprises the addition of a controlled amount of an affinity molecule to said effluent, whereby the affinity molecules bind analytes in the effluent, followed by a separation step using a restricted-access support, whereby the analyte-affinity molecule complex is permeated, followed by a suitable dissociation step to dissociate the analyte-affinity molecule complex, followed by a second separation step in which the dissociated analyte and affinity molecules are separated, followed by detection of the analyte using the mass spectrometer.

2. On-line detection method according to claim 1, in which the second separation step is carried out using a restricted-access support, in which the affinity molecule is retained, followed by elution of the analyte from the restricted-access support using a suitable carrier stream, and directing the eluted stream to the mass spectrometer.

3. On-line detection method according to claim 1, in which the second separation step is carried out using a hollow fiber support, whereby the analyte is permeated and the permeate is directed to the mass spectrometer.

4. On-line detection method according to any of the preceding claims, in which the dissociation step is a low pH shock, contacting with a high ionic strength solution, contacting with an organic solvent and/or contacting with a chaotropic reagent.

5. Method according to any of the preceding claims in which the fractionation step is a liquid chromatography separation, a capillary electrophoresis step or a

(61)

Title: Orphan receptor assay using on-line coupling of mass spectrometry to continuous-flow separation techniques.

Abstract

The present invention relates to the on-line coupling of mass spectrometry (MS) to continuous-flow separation techniques for detecting 'orphan' analytes, viz. receptors which do not have known ligands. In a further embodiment, this on-line detection method is used as either a screening method for assays with an unknown ligand. Furthermore, this invention relates to compounds detected by this method and the use of these compounds as a ligand for affinity molecules.

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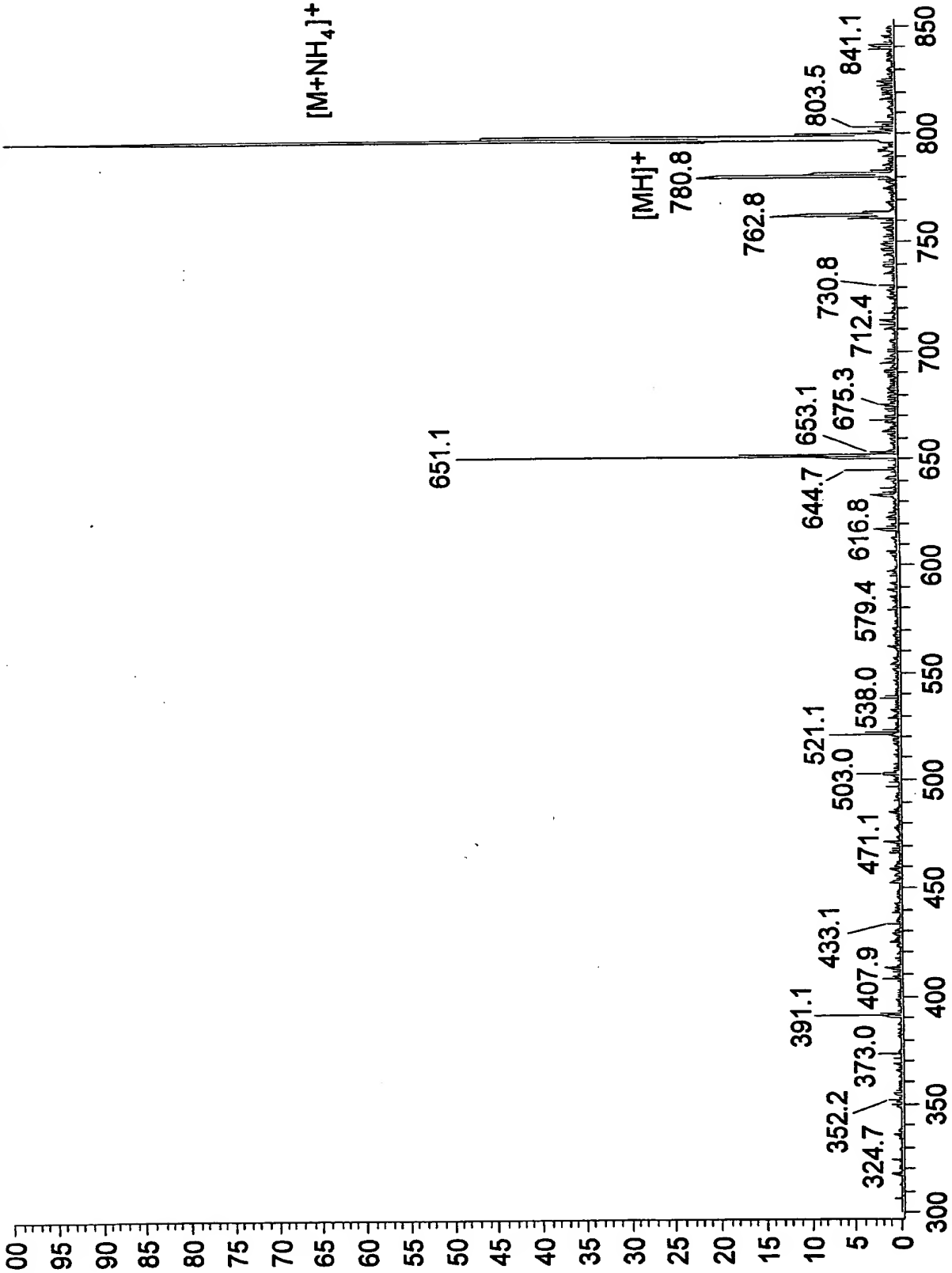
FIG. 1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